Practitioner's Docket No. MPI01-021P1RNM *PATENT*

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	Glucksmann, Maria A.		
Application No.:	10/085233	Group No.:	1646
Filed:	February 28, 2002	Examiner:	Basi, Nirmal Singh
For:	93870, A HUMAN G-PROTEIN COUPLED RECEPTOR AND USES THEREFOR		

Mail Stop Amendment
Commissioner for Patents
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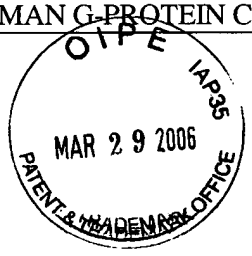
Alexandria, VA 22313-1450



DECLARATION UNDER 37 C.F.R. § 1.132

Sir:

I, Timothy Ocain, of 45 Indian Head Road, Framingham, Massachusetts 01701, hereby declare and state:

1. I am currently employed by Millennium Pharmaceuticals, Inc. as the Senior Director of Inflammation. My curriculum vitae is attached as Exhibit A.

2. The above-identified patent application was filed on behalf of Millennium Pharmaceuticals, Inc., 40 Landsdowne Street, Cambridge, Massachusetts 02139, the Assignee of record. This patent application claims priority to U.S. Provisional Application Serial No. 60/272,677, filed March 1, 2001.

CERTIFICATION UNDER 37 C.F.R. SECTIONS 1.8(a) and 1.10*

I hereby certify that, on the date shown below, this correspondence is being:

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Date: March 27, 2006

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3. I am the supervisor of the Inflammation Discovery program at Millennium and was involved in reviewing 93870 for inclusion in our discovery program. I am familiar with the application, the invention claimed therein and have reviewed the rejection of these claims under 35 U.S.C. §101, set forth in the Office Action mailed from the U.S. Patent and Trademark Office on September 27, 2005.

4. The Examiner states that claims 28-29 and 32-43 are not supported by either a specific and substantial asserted utility or a well established utility. Specifically, the Examiner states that Applicant has asserted utilities for the specifically claimed invention of claims 28-29 and 32-43, as drawn to a method of identifying compounds that bind to 93870 polypeptide having the amino acid sequence disclosed in SEQ ID NO:2, which is encoded by the nucleic acids disclosed in SEQ ID NO:1 and SEQ ID NO:3 and that 93870 polypeptide is disclosed to have structural characteristics of a G protein-coupled receptor. The Examiner further states that the specification does not disclose further listed details to support this identification. One of the details considered lacking is specific treatable diseases associated with the GPCR of SEQ ID NO:2. I note that in paragraph [0055], the specification discloses that:

"93870 molecules and modulators thereof can act as novel therapeutic agents for controlling one or more of GPCR associated disorders, e.g., immune and inflammatory disorders"

The specification, in paragraph [0056] further lists examples of specific immune and inflammatory disorders. Herewith, I am providing evidence which supports these statements, that the 93870 GPCR plays a role in immune and inflammatory disorders, as disclosed in the specification.

5. Enclosed as Exhibit B is expression data which shows that 93870 expression is consistent with its involvement in immune and inflammatory diseases, including rheumatoid arthritis and inflammatory bowel diseases, and also potentially chronic obstructive pulmonary disease. Exhibit B consists of slides which were presented by a Millennium scientist, Ethan Grant at a meeting held in July 2002. These slides provide results of expression studies in support of a decision to include 93870 as a target in an Inflammation Discovery Program at Millennium. They culminate several studies, including *in vivo* studies, which were performed during an extended period previous to that date. I reviewed these data at the time and I was involved in the decision to include 93870 in the Inflammation Discovery program, in part, due to these data. I hereby verify the validity of this data and the summary statements provided by Dr. Grant.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Timothy Ocain

3-23-06

Timothy Ocain Date

Docket No. MPI01-021P1RNM

U.S. Serial No. 10/085,233, Filed February 28, 2002

Exhibits A, B and C Accompanying Declaration under 37 CFR 1.132 and Amendment and Response to Office Action Dated September 27, 2005

Exhibit A	Curriculum Vitae of Timothy Ocain
Exhibit B	Expression Data
Exhibit C	Wilson, Shelagh, et al., "Orphan G-protein-coupled receptors: the next generation of drug targets?" *British Journal of Pharmacology*, Vol. 125 (1998) pp 1387-1392

Timothy D. Ocain, Ph.D.

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Professional Experience

Millennium Pharmaceuticals, Inc. **Cambridge, MA** **1998-present**

Senior Director, Inflammation Discovery **2002-present**

Major line responsibility (35-65 people, >10 MM budget) for managing pharmacology/cellular immunology/biochemistry efforts and interfacing with chemistry, DMPK, and drug safety with primary goal of delivering development candidates for Inflammation franchise.

- Primary accomplishments: Principal role in the delivery of multiple development candidates (MLN3316, MLN3897, MLN3701, MLN0415, MLN6095) as well as many additional advanced candidate-seeking compounds in the areas of rheumatoid arthritis, asthma, COPD, multiple sclerosis, and other inflammatory conditions.
- Ongoing (5+ yrs) chair of Joint Research Subcommittee for Millennium-Aventis (sanofi-aventis) collaboration.
- Core member of multiple internal tactical and strategic committees, including: Inflammation Strategic Business Team, Pipeline Review Committee, Cardiovascular/Inflammation Advisory Group, Discovery Scientific Review Committee. Includes interfacing with finance and business development groups.

Director ('00-'02), Senior Director ('02), Inflammation Program Management **2000-2002**

Matrix manager of Inflammation efforts involving over 15 departments, including genomics, disease biology and pharmacology, chemistry, high throughput screening, etc.

- Integral role in design and implementation of unique broad-based 50:50 co-discovery co-development alliance with Aventis, focusing on small molecule modulators for inflammatory conditions. Chair of Joint Research Subcommittee overseeing all aspects of the alliance (>400 fte's across both companies at peak). Also a member of Joint Development subcommittee.
- Interfaced with finance and line heads for budgets, business plans, value creation, and productivity models within Inflammation. Principal role in M&A and in-licensing assessments.

Associate Director, Drug Discovery **1998-2000**

Major drug discovery responsibilities for emerging small-molecule pipeline; line management (including major personnel and infrastructure build-up) for medicinal chemistry department.

- Designed MLN4760, first small molecule clinical compound at MLNM targeting a gene discovered via internal genomics efforts.
- Member of research steering committees and primary drug discovery representative for three major alliances focused on leveraging Millennium genomic discoveries and early drug discovery expertise to contribute to large pharma pipelines and the initiation of new chemistry programs: antifungal alliance with Pfizer, antibacterial alliance with Wyeth, and CNS alliance with Wyeth.

Procept, Inc. **Cambridge, MA** **1993-1998**

Vice President, Chemistry and Structural Biology **1997-1998**
Director, Chemistry and Structural Biology **1996-1997**
Group Leader, Medicinal Chemistry **1993-1996**

As Director and VP, responsible for directing all medicinal chemistry, analytical chemistry, and structural biology (high-field NMR, X-ray, computational) activities directed at discovering new molecules to block immunological and infectious disease targets. Extensive involvement in biology, DMPK, and safety for all drug discovery programs.

- Led group that discovered and delivered PRO2000, the lead HIV clinical candidate, provided discovery support for parental and topical first-in-human studies in Europe (currently in PhII trials).

Wyeth-Ayerst Research **Princeton, NJ** **1986-1993**

Principal Scientist, Immunology/Inflammation Medicinal Chemistry **1992-1993**
Research Scientist, Immunology/Inflammation Medicinal Chemistry **1988-1992**
Key involvement in new initiatives at W-A, e.g.: integration of structural biology and computational chemistry with drug discovery; collaboration with biotechnology; discovery-based interactions with DMPK group.

- Project Leader of Rapamycin Immunomodulation Project. Led efforts of large discovery team, delivered advanced lead compounds with appropriate in vivo, DMPK, and metabolic profile suitable for non-clinical development. Extensive interaction with outside collaborators.
- Responsible for discovery support of oral IND phase for novel marketed immunosuppressant drug, Rapamune®.

Senior Chemist, Cardiovascular /CNS Medicinal Chemistry **1986-1988**
Project leader of Renin Project and Renin Processing Enzyme Project, delivered advanced lead compound for primate efficacy and toxicology studies.

Education/Post-doctoral Studies

1986, Post-Doctoral Fellow. University of Minnesota, Chemistry, under the direction of Professor Thomas R. Hoye: Total Synthesis of anti-tumor natural product Uvaricin.

1985, Ph.D., Pharmaceutical Chemistry, University of Wisconsin-Madison, under the direction of Professor Daniel H. Rich: Design, synthesis and enzymatic evaluation of metalloprotease inhibitors.

1981, University of Wisconsin-Eau Claire, B.S. Biological Sciences: 2 years undergraduate medicinal chemistry research on serine protease inhibitors.

Publications

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Liu, S., Neidhardt, E.A., Grossman, T.H., Ocain, T., and Clardy, J., "Structures of Human Dihydroorotate Dehydrogenase in Complex with Antiproliferative Agents," *Structure*, 2000, *8*, 25-33.

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Ocain, T. D. and Deininger, D. D. "Renin Inhibitors," US 5,023,338 (1991).

93870 Summary

• 93870 is a nameless, orphan GPCR. It was identified as a potential target based on expression analyses done in conjunction with Molecular Pathology (Genomics A□). It has not been described in any publications in the literature.

• The mouse and human sequences are 74% identical at the protein level; there is a short C-terminal region that is highly divergent between the two receptors.

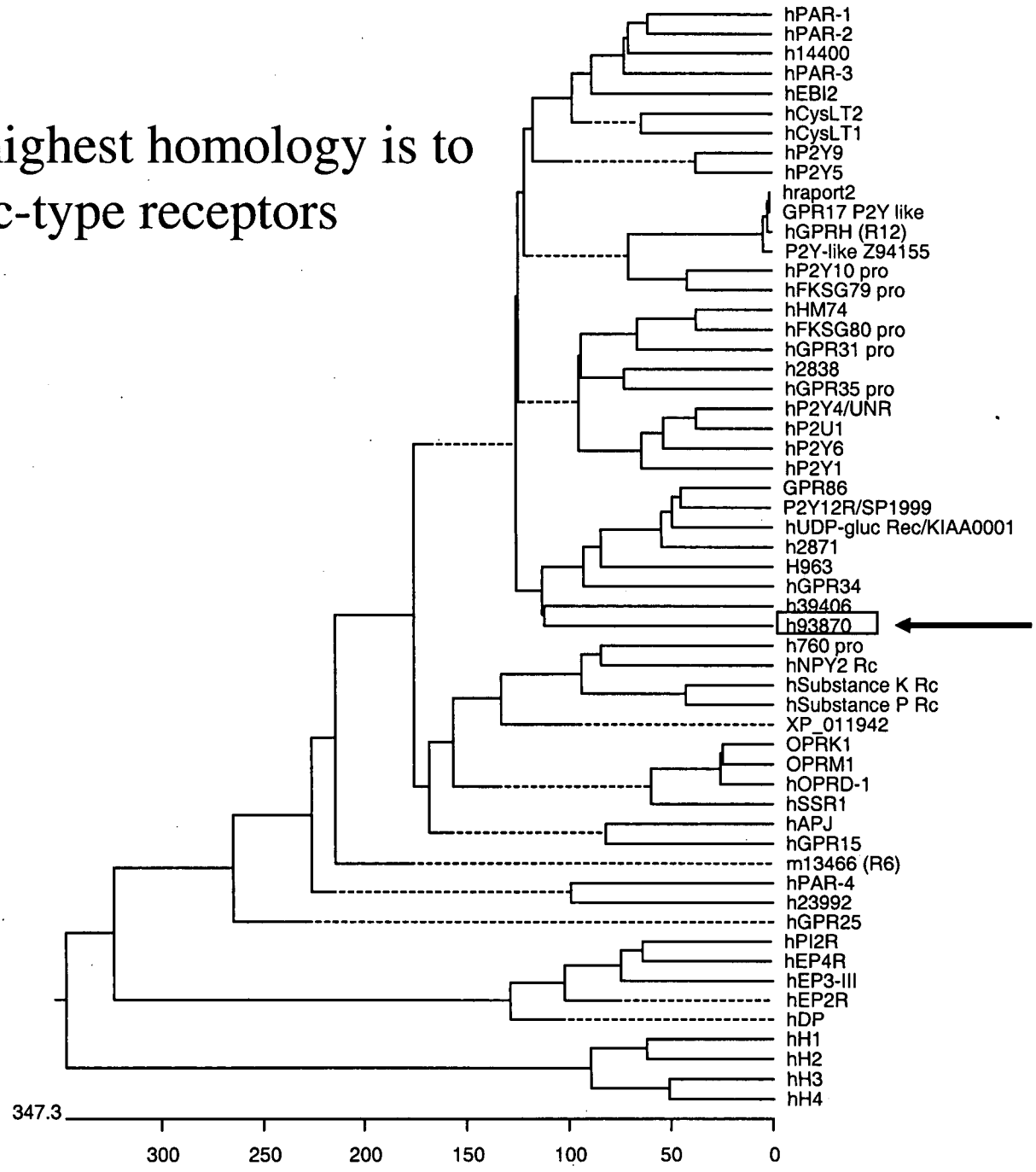
• BLAST searches with the mouse protein sequence indicates some weak homology to CCR1, GPR34 and other GPCRs.

• Protein sequence alignments with the human receptor suggest that the highest homology is with purinergic GPCRs, providing a rational de-orphaning strategy.

• Expression is highest in monocytes, macrophages and granulocytes. Expression was detected in most RA tissues, showing a pattern similar to that observed for the control gene CD68, consistent with expression of the target in tissue macrophages and/or granulocytes. Expression is upregulated in both CIA and ABIA models of arthritis.

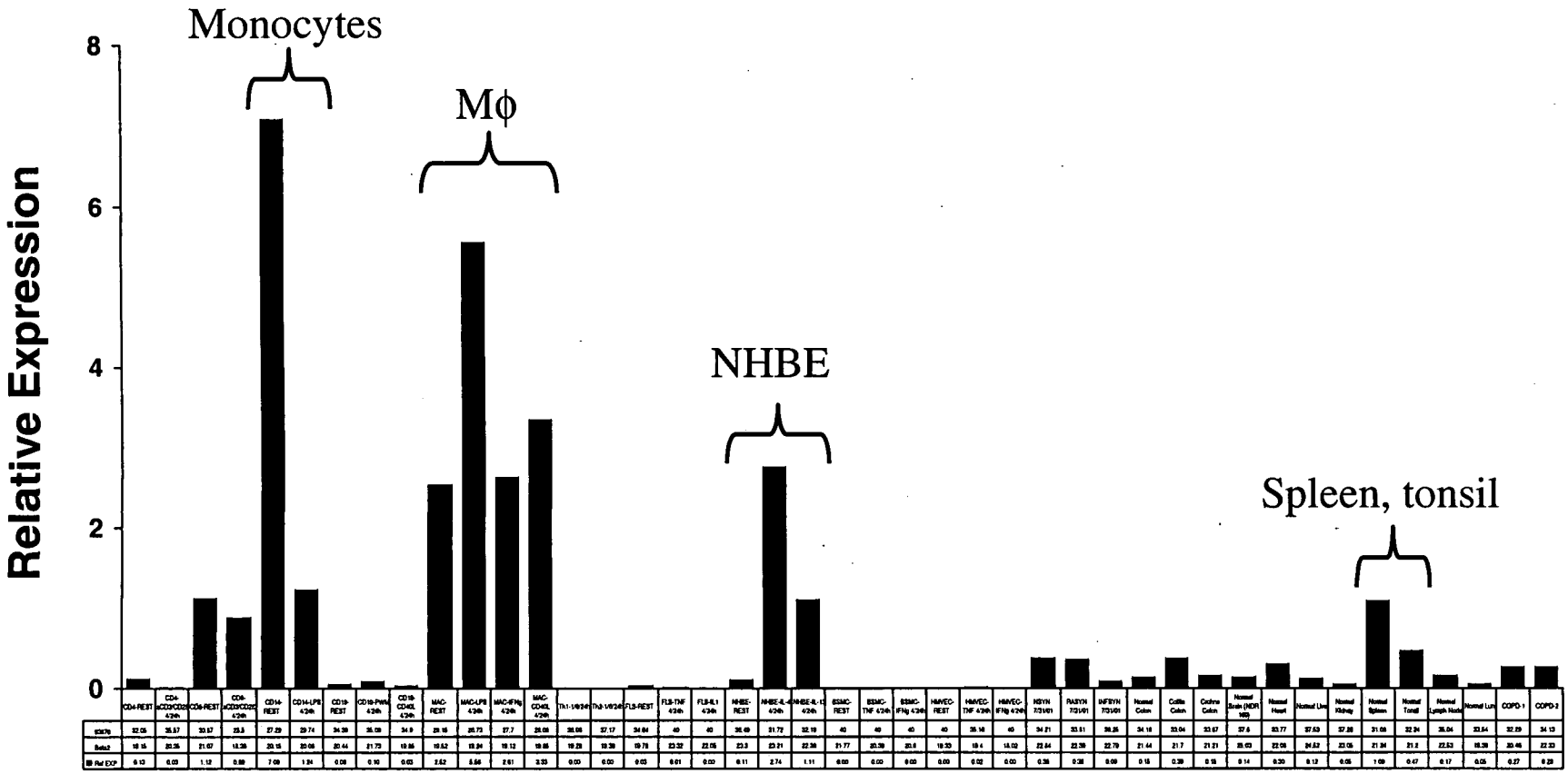
• Higher expression was detected in human UC tissues compared to normal colons and in a mouse model of IBD. Little to no expression was detected in normal or COPD lung tissue, though the expression in monocytes, macrophages and activated NHBE cells makes the gene of interest for further analyses of human COPD and mouse model tissues when additional reagents become available.

GPCR 93870: highest homology is to purinergic-type receptors

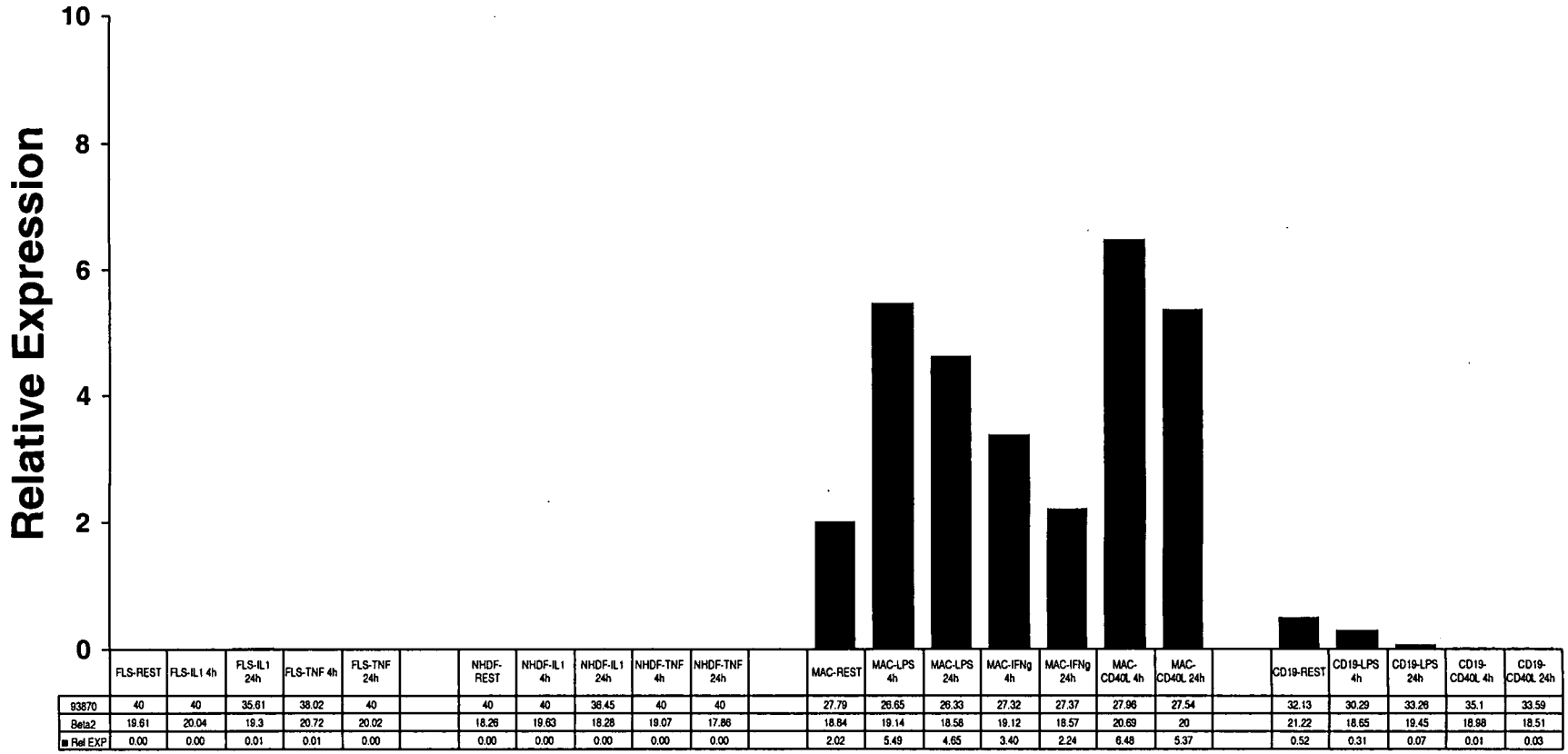


Ethan P Grant

93870 Expression: Human cells and tissues



93870 Expression: Human cells



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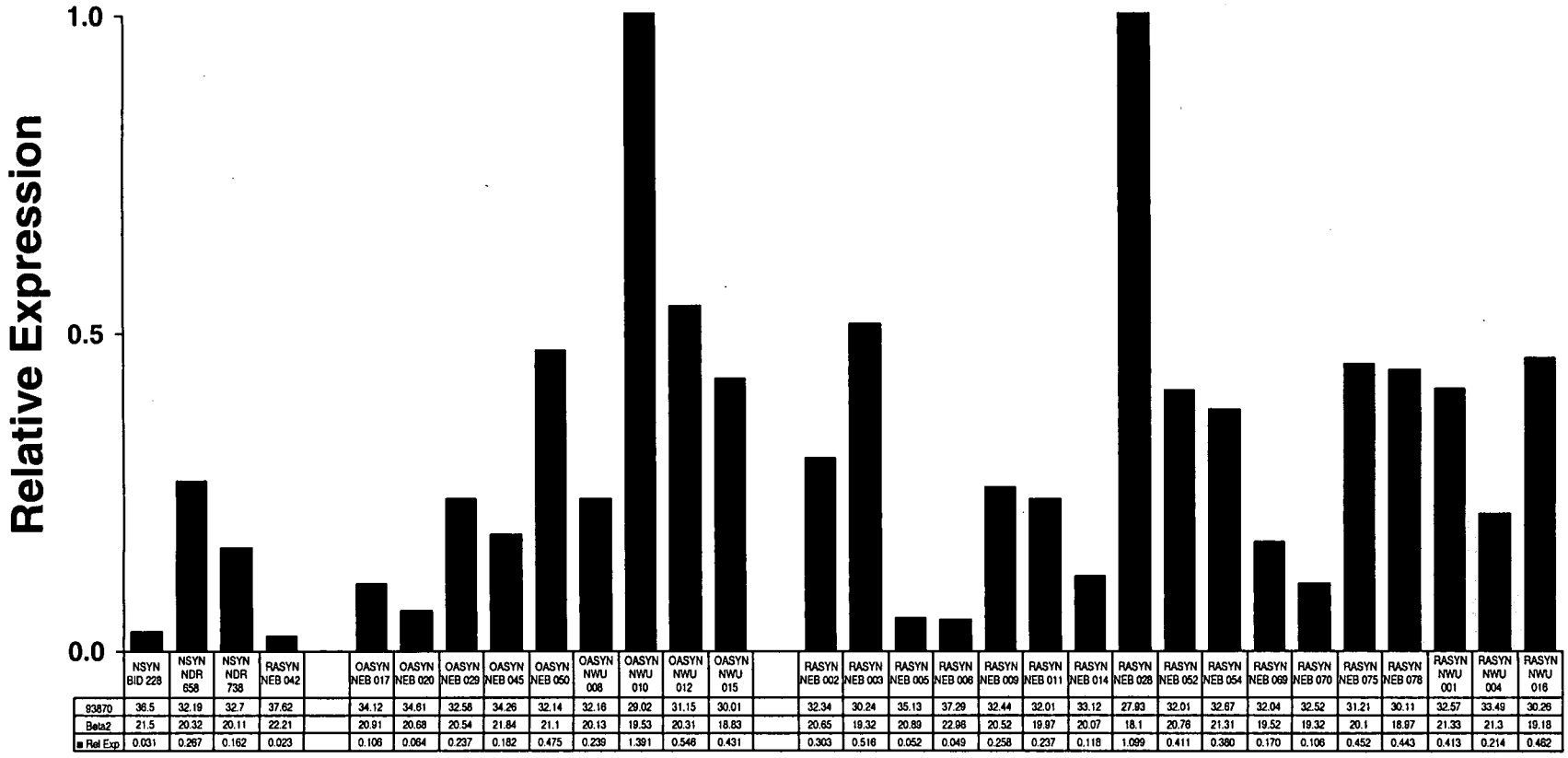
93870	40	40	35.61	38.02	40		40	40	38.45	40	40		27.79	26.65	26.33	27.32	27.37	27.96	27.54		32.13	30.29	33.26	35.1	33.59
Beta2	19.61	20.04	19.3	20.72	20.02		18.26	19.63	18.28	19.07	17.86		18.84	19.14	18.58	19.12	18.57	20.69	20		21.22	18.65	19.45	18.98	18.51
Rel EXP	0.00	0.00	0.01	0.01	0.00		0.00	0.00	0.00	0.00	0.00		2.02	5.49	4.65	3.40	2.24	6.48	5.37		0.52	0.31	0.07	0.01	0.03

FLS **NHDF** **Macrophages** **B cells**

93870 Expression: Human synovium



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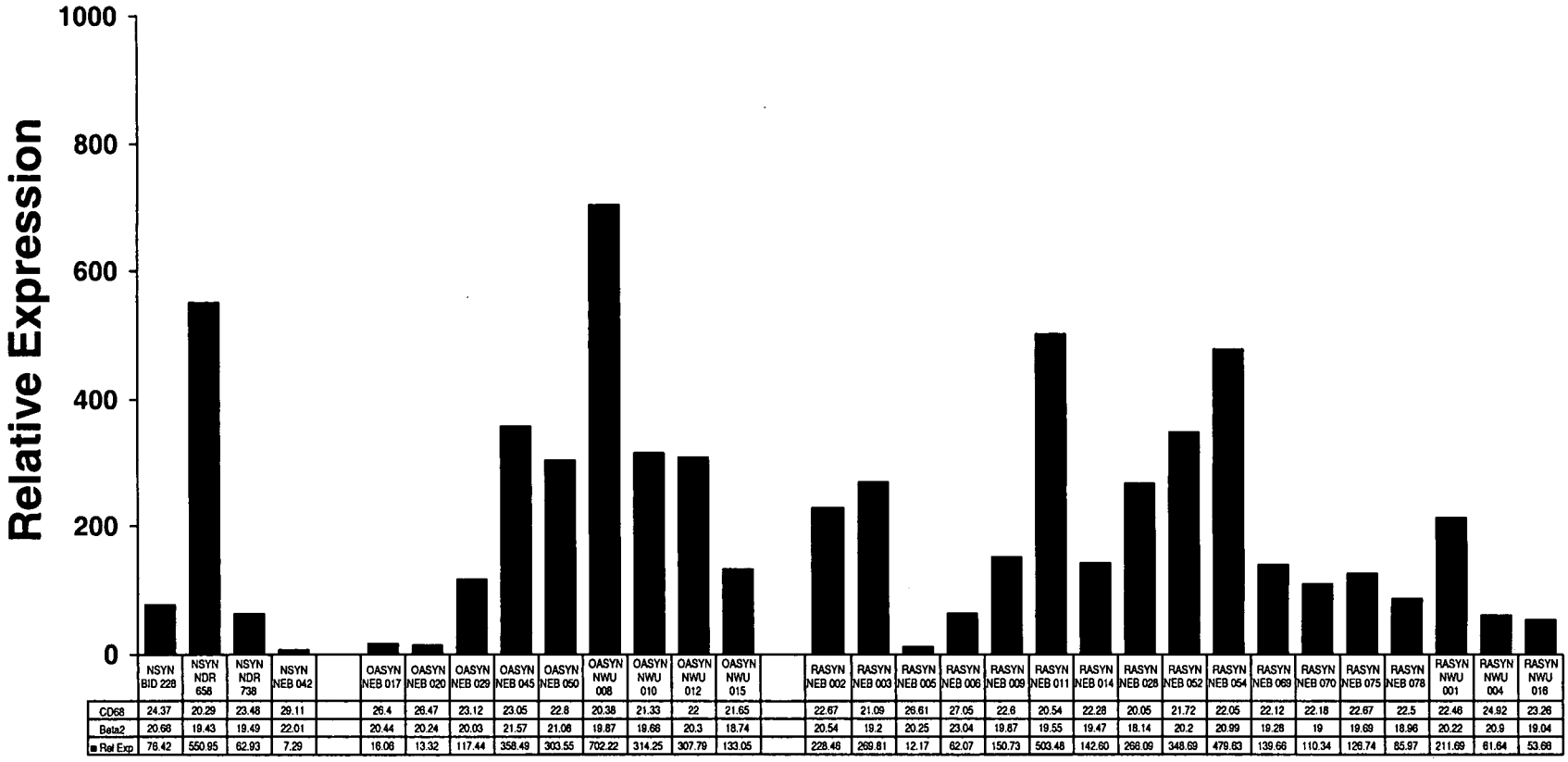
93870	36.5	32.19	32.7	37.62		34.12	34.61	32.58	34.26	32.14	32.16	29.02	31.15	30.01		32.34	30.24	35.13	37.29	32.44	32.01	33.12	27.93	32.01	32.67	32.04	32.52	31.21	30.11	32.57	33.49	30.26
Beta2	21.5	20.32	20.11	22.21		20.91	20.68	20.54	21.84	21.1	20.13	19.53	20.31	18.83		20.65	19.32	20.89	22.96	20.52	19.97	20.07	18.1	20.76	21.31	19.52	19.32	20.1	18.97	21.33	21.3	19.18
Rel Exp	0.031	0.267	0.162	0.023		0.106	0.064	0.237	0.182	0.475	0.239	1.391	0.546	0.431		0.303	0.516	0.052	0.049	0.258	0.237	0.118	1.099	0.411	0.380	0.170	0.106	0.452	0.443	0.413	0.214	0.462

Normal OA RA

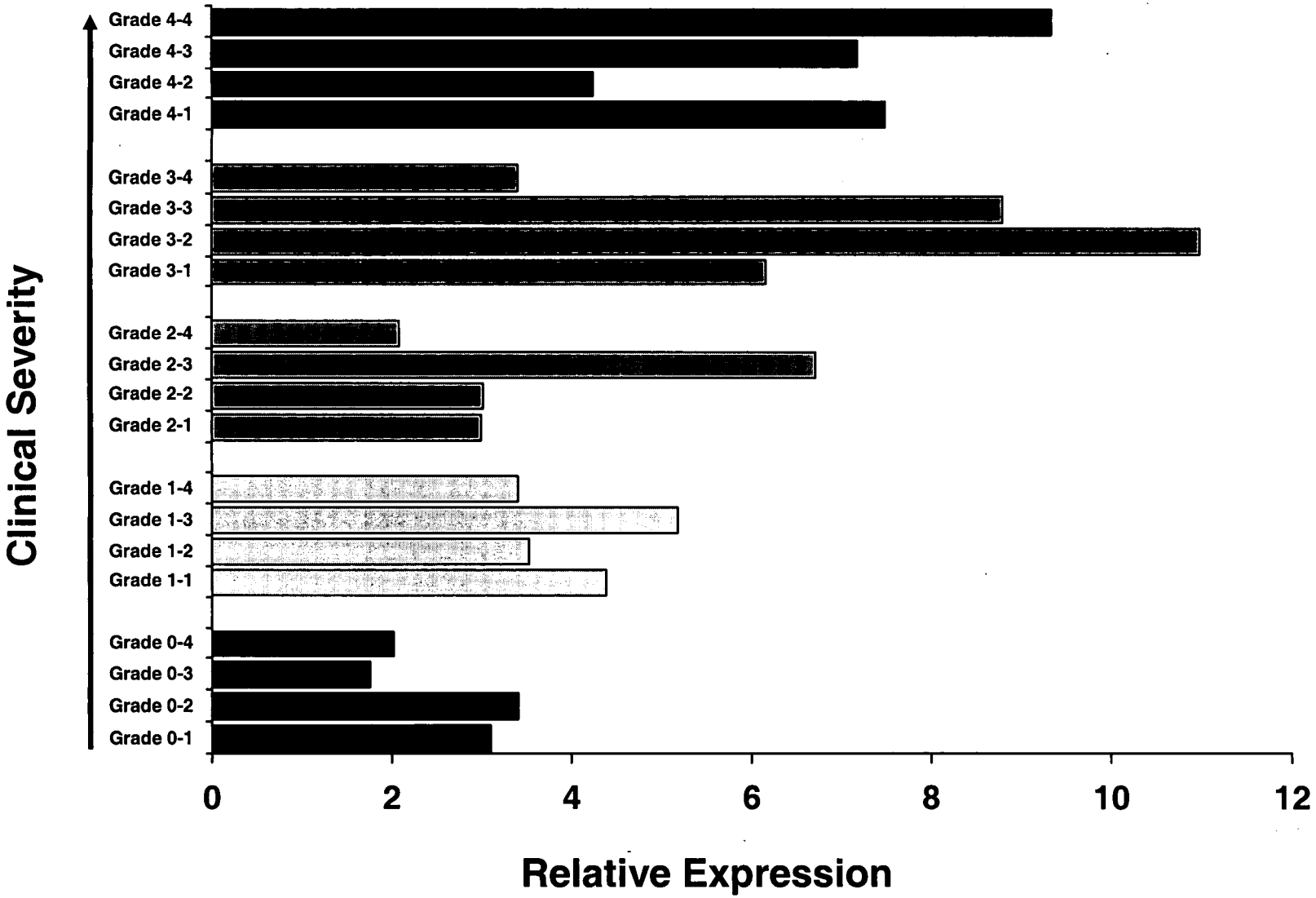
CD68 Expression: Human synovium



	NSYN BID 228	NSYN NDR 658	NSYN NDR 738	NSYN NEB 042		OASYN NEB 017	OASYN NEB 020	OASYN NEB 029	OASYN NEB 045	OASYN NEB 050	OASYN NWU 008	OASYN NWU 010	OASYN NWU 012	OASYN NWU 015		RASYN NEB 002	RASYN NEB 003	RASYN NEB 005	RASYN NEB 006	RASYN NEB 009	RASYN NEB 011	RASYN NEB 014	RASYN NEB 028	RASYN NEB 052	RASYN NEB 054	RASYN NEB 069	RASYN NEB 070	RASYN NEB 075	RASYN NEB 078	RASYN NWU 001	RASYN NWU 004	RASYN NWU 016
CD68	24.37	20.29	23.48	29.11		26.4	26.47	23.12	23.05	22.8	20.38	21.33	22	21.65		22.67	21.09	26.61	27.05	22.8	20.54	22.28	20.05	21.72	22.05	22.12	22.18	22.67	22.5	22.46	24.92	23.26
Beta2	20.68	19.43	19.49	22.01		20.44	20.24	20.03	21.57	21.08	19.87	19.66	20.3	18.74		20.54	19.2	20.25	23.04	19.87	19.55	19.47	18.14	20.2	20.99	19.28	19	19.69	18.96	20.22	20.9	19.04
Rel Exp	76.42	550.95	62.93	7.29		16.06	13.32	117.44	358.49	303.55	702.22	314.25	307.79	133.05		228.48	269.81	12.17	62.07	150.73	503.48	142.60	268.09	348.69	479.63	139.66	110.34	126.74	85.97	211.69	61.64	53.68

Normal OA RA

93870 Expression: Mouse CIA Model



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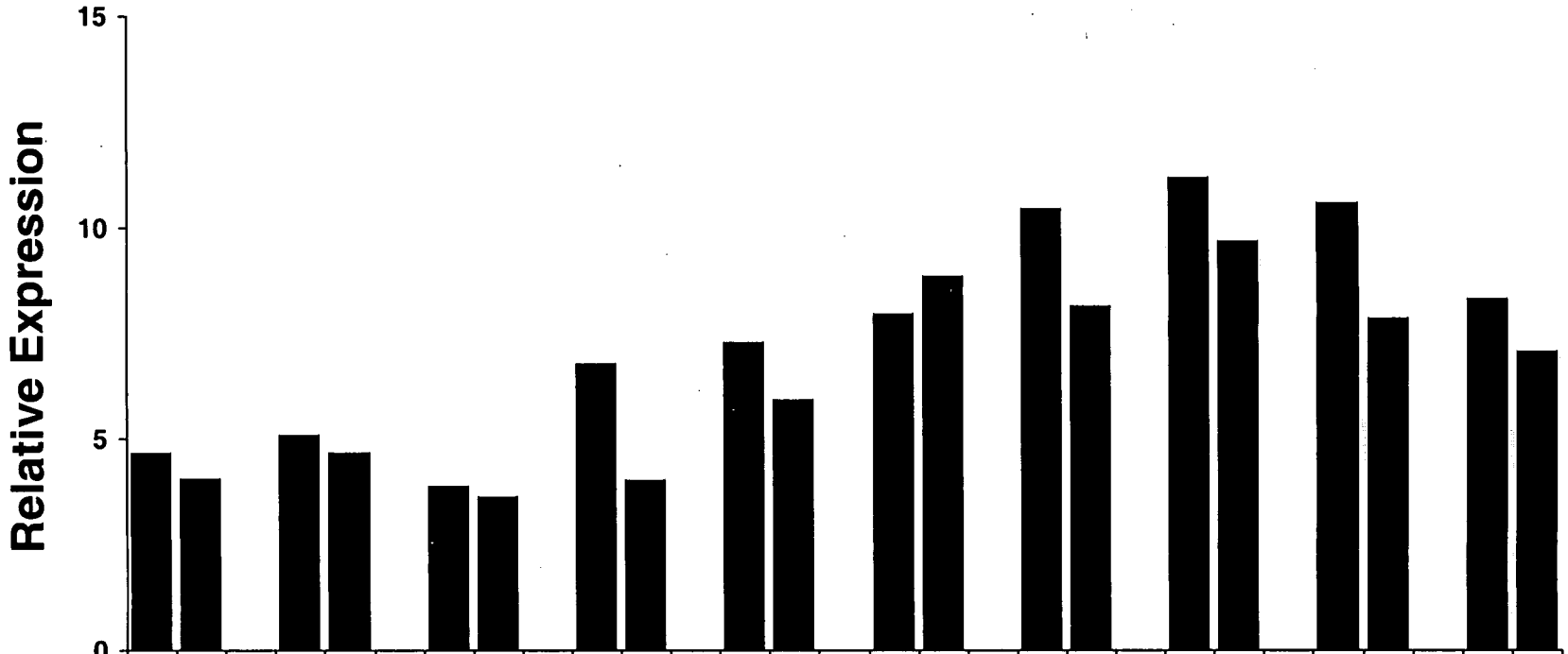

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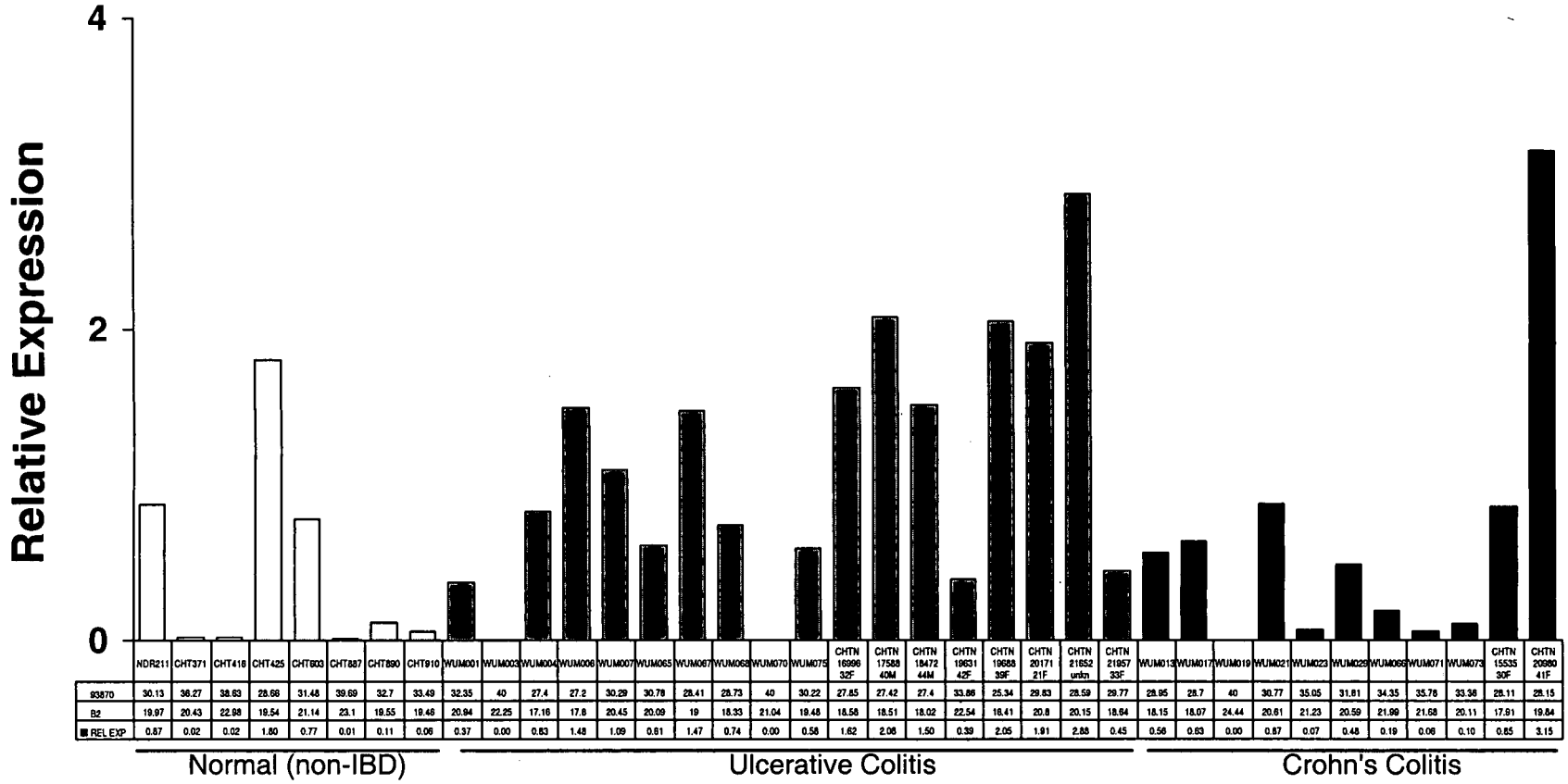
m93870	27.26	28.11		27.29	27.86		27.63	27.78		27.26	27.42		26.79	27.21		26.92	27.75		26.68	27.27		26.69	27.17		27.07	27.43		27.23	27.65
GAPDH	19.52	20.18		19.68	20.12		19.63	19.68		20.06	19.47		19.69	19.81		19.95	20.93		20.1	20.33		20.21	20.48		20.51	20.44		20.32	20.51
REL EXP	4.68	4.10		5.12	4.68		3.91	3.64		6.80	4.04		7.29	5.92		7.98	8.85		10.45	8.14		11.20	9.69		10.60	7.87		8.32	7.09

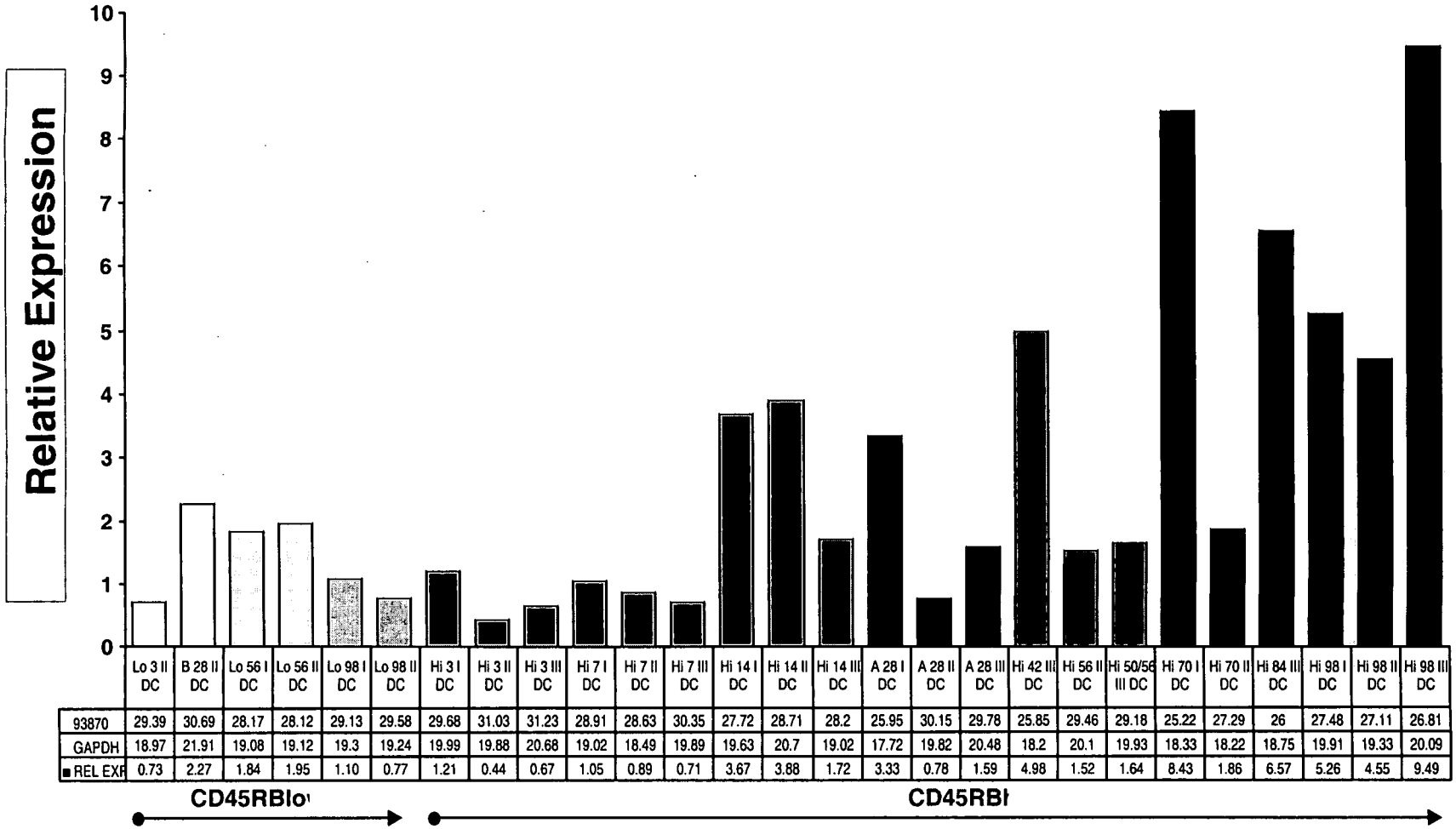
Time post antibody transfer

93870 Expression: IBD Colons



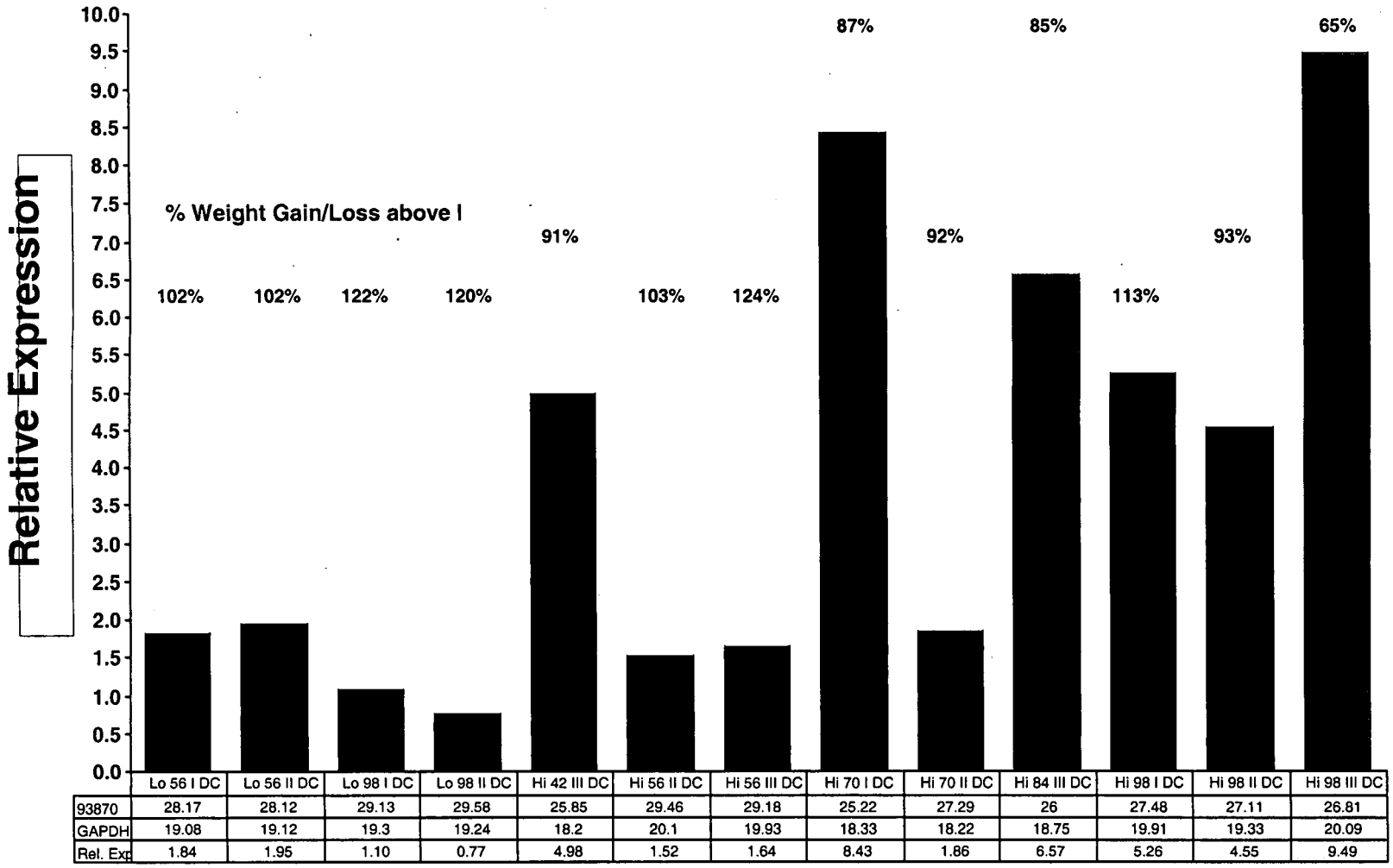
	NDR211	CHT371	CHT416	CHT425	CHT603	CHT887	CHT890	CHT910	WUM001	WUM003	WUM004	WUM006	WUM007	WUM065	WUM067	WUM068	WUM070	WUM075	CHTN 16996 32F	CHTN 17588 40M	CHTN 18472 44M	CHTN 19631 42F	CHTN 19688 39F	CHTN 20171 21F	CHTN 21652 unkn	CHTN 21957 33F	WUM013	WUM017	WUM019	WUM021	WUM023	WUM029	WUM066	WUM071	WUM073	CHTN 15535 30F	CHTN 20980 41F
93870	30.13	36.27	38.63	28.66	31.48	39.69	32.7	33.49	32.35	40	27.4	27.2	30.29	30.78	28.41	28.73	40	30.22	27.85	27.42	27.4	33.86	25.34	29.83	28.59	29.77	28.95	28.7	40	30.77	35.05	31.81	34.35	35.78	33.36	28.11	28.15
B2	19.97	20.43	22.98	19.54	21.14	23.1	19.55	19.48	20.94	22.25	17.16	17.8	20.45	20.09	19	18.33	21.04	19.48	18.58	18.51	18.02	22.54	18.41	20.8	20.15	18.64	18.15	18.07	24.44	20.61	21.23	20.59	21.99	21.68	20.11	17.91	19.84
REL EXP	0.87	0.02	0.02	1.80	0.77	0.01	0.11	0.06	0.37	0.00	0.83	1.48	1.09	0.81	1.47	0.74	0.00	0.58	1.62	2.08	1.50	0.39	2.05	1.91	2.88	0.45	0.56	0.63	0.00	0.87	0.07	0.48	0.19	0.08	0.10	0.85	3.15

m93870 Expression in murine IBD Phas



	Lo 3 II DC	B 28 II DC	Lo 56 I DC	Lo 56 II DC	Lo 98 I DC	Lo 98 II DC	Hi 3 I DC	Hi 3 II DC	Hi 3 III DC	Hi 7 I DC	Hi 7 II DC	Hi 7 III DC	Hi 14 I DC	Hi 14 II DC	Hi 14 III DC	A 28 I DC	A 28 II DC	A 28 III DC	Hi 42 III DC	Hi 56 II DC	Hi 50/56 III DC	Hi 70 I DC	Hi 70 II DC	Hi 84 III DC	Hi 98 I DC	Hi 98 II DC	Hi 98 III DC
93870	29.39	30.69	28.17	28.12	29.13	29.58	29.68	31.03	31.23	28.91	28.63	30.35	27.72	28.71	28.2	25.95	30.15	29.78	25.85	29.46	29.18	25.22	27.29	26	27.48	27.11	26.81
GAPDH	18.97	21.91	19.08	19.12	19.3	19.24	19.99	19.88	20.68	19.02	18.49	19.89	19.63	20.7	19.02	17.72	19.82	20.48	18.2	20.1	19.93	18.33	18.22	18.75	19.91	19.33	20.09
■ REL EXP	0.73	2.27	1.84	1.95	1.10	0.77	1.21	0.44	0.67	1.05	0.89	0.71	3.67	3.88	1.72	3.33	0.78	1.59	4.98	1.52	1.64	8.43	1.86	6.57	5.26	4.55	9.49

m93870 Expression in Select IBD Phas



	Lo 56 I DC	Lo 56 II DC	Lo 98 I DC	Lo 98 II DC	Hi 42 III DC	Hi 56 II DC	Hi 56 III DC	Hi 70 I DC	Hi 70 II DC	Hi 84 III DC	Hi 98 I DC	Hi 98 II DC	Hi 98 III DC
93870	28.17	28.12	29.13	29.58	25.85	29.46	29.18	25.22	27.29	26	27.48	27.11	26.81
GAPDH	19.08	19.12	19.3	19.24	18.2	20.1	19.93	18.33	18.22	18.75	19.91	19.33	20.09
Rel. Exp	1.84	1.95	1.10	0.77	4.98	1.52	1.64	8.43	1.86	6.57	5.26	4.55	9.49

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REVIEW ARTICLE

Orphan G-protein-coupled receptors: the next generation of drug targets?

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The pharmaceutical industry has readily embraced genomics to provide it with new targets for drug discovery. Large scale DNA sequencing has allowed the identification of a plethora of DNA sequences distantly related to known G protein-coupled receptors (GPCRs), a superfamily of receptors that have a proven history of being excellent therapeutic targets. In most cases the extent of sequence homology is insufficient to assign these 'orphan' receptors to a particular receptor subfamily. Consequently, reverse molecular pharmacological and functional genomic strategies are being employed to identify the activating ligands of the cloned receptors. Briefly, the reverse molecular pharmacological methodology includes cloning and expression of orphan GPCRs in mammalian cells and screening these cells for a functional response to cognate or surrogate agonists present in biological extract preparations, peptide libraries, and complex compound collections. The functional genomics approach involves the use of 'humanized yeast cells, where the yeast GPCR transduction system is engineered to permit functional expression and coupling of human GPCRs to the endogenous signalling machinery. Both systems provide an excellent platform for identifying novel receptor ligands. Once activating ligands are identified they can be used as pharmacological tools to explore receptor function and relationship to disease.

Keywords: Orphan; novel receptor; 7TM receptor; ligand fishing; novel peptide

Introduction

Rapid advances in DNA sequencing technologies have led to an exponential increase in the generation of genomic information. Such information holds enormous potential for drug discovery, allowing the identification of a diverse range of novel molecular targets. The challenge for the pharmaceutical industry in dealing with this wealth of new information is to identify the most promising candidates for further biological evaluation and to characterize their potential as drug targets as rapidly and efficiently as possible. This review will focus on one family of novel molecular targets, the family of orphan G-protein coupled receptors (GPCRs) and describe the strategies one can adopt to convert them into therapeutically relevant drug targets.

The superfamily of GPCRs is one of the largest families of genes yet identified. Over 800 members have been cloned to date from a wide range of species. The characteristic motif of this superfamily is the seven distinct hydrophobic regions, each 20–30 amino acids in length, generally regarded as the transmembrane domains of these integral membrane proteins. There is little conservation of amino acid sequence across the entire superfamily of receptors, but key sequence motifs can be found within phylogenetically related sub-families, and these motifs can be used to help classify new members.

Since the first cloning of GPCR cDNAs more than a decade ago, new genes have continued to emerge whose sequences place them firmly within the GPCR superfamily, but whose ligands remain to be identified. These 'orphan' receptors show low levels of homology with known GPCRs (typically less than 40%), too low to classify them with any confidence into a specific receptor subfamily. Many orphan receptors in fact show closer homology to each other than to known GPCRs, suggesting that they may represent new sub-families of receptors with distinct, possibly novel, ligands. These sub-families are distributed throughout the GPCR superfamily tree, suggesting that they will have a diverse range of functions.

What is the rationale for investing resources in characterizing orphan GPCRs? Simply stated, GPCRs have a proven history of being excellent drug targets. Several hundred drugs launched in the last three decades are directed at known GPCRs. Table 1 shows a representative snapshot of some well-established drugs and their corresponding receptors. It is clear that the therapeutic focus of these drugs spans a wide range of disorders from cardiovascular to gastro-intestinal to CNS and others.

Another example of the significance of GPCRs to drug discovery involves the increasing number of diseases associated with receptor gene mutations (Table 2). Some of the early examples identified involved mutations in receptors which caused gross changes in receptor function, leading to fairly rare but severe inherited disorders (e.g., mutations in vasopressin V_2 receptors associated with X-linked nephrogenic diabetes insipidus); (Birnbaumer, 1995). More recently, mutations have been identified that cause little or no apparent change in receptor function which lead merely to an increased propensity for developing a multifactorial disease (e.g. polymorphisms in the β_3-adrenoceptor associated with increased risk of insulin resistance); (Strosberg, 1997). As genotyping of disease populations becomes more comprehensive it is likely that more GPCR mutations or polymorphisms will be associated with disease states and hence provide additional potential targets for drug intervention. Since knowledge of gene function is not a pre-requisite for carrying out genotyping studies, it is just as likely that such mutations will be found in orphan receptors, which may provide clues to orphan receptor function in physiological and pathophysiological situations.

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Table 1 Drugs targeting GPCRs

GPCR	Generic	GPCR	Generic
Acetylcholine	Bethanechol Dicyclomine Ipratropium	Leukotriene	Pranlukast Zafirlukast
Adrenoceptor	Atenolol Clonidine Propranolol Terazosin Albuterol Carvedilol	Opioid	Buprenorphine Butorphanol Alfentanil Morphine
Angiotensin II	Losartan Eprosartan	Prostaglandin	Epoprostenol Misoprostol
Dopamine	Metoclopramine Ropinirole Haloperidol	Somatostatin	Octreotide
Histamine	Dimenhydrinate Terfenadine Cimetidine Ranitidine	Serotonin	Sumatriptan Ritanserin Cisapride Trazodone Clozapine

Table 2 Diseases associated with GPCR mutations

Receptor	Disease
Rhodopsin	Retinitis pigmentosa
Thyroid stimulating hormone	Hyperfunctioning thyroid adenomas
Lutenizing hormone	Precocious puberty
Vasopressin V_2	X-linked nephrogenic diabetes
Calcium	Hyperparathyroidism, hypocalciuria, hypercalcemia
Parathyroid hormone	Short limbed dwarfism
β_3-Adrenoceptor	Obesity, NIDDM
Growth hormone releasing hormone	Dwarfism
Adrenocorticotropin	Glucocorticoid deficiency
Glucagon	Diabetes, hypertension

Much current research effort within the pharmaceutical industry today continues to focus on GPCRs, as they are justifiably perceived as attractive therapeutic targets. At SmithKline Beecham we have identified over 100 human orphan GPCRs that are distributed throughout the GPCR evolutionary tree. Some of these receptors are selectively expressed in a range of therapeutically relevant tissues, and it thus seems reasonable to speculate that they constitute a source of therapeutic targets with similar potential for drug discovery as seen with known GPCRs.

Reverse pharmacology approach

The overall strategy for characterizing orphan receptors has often been referred to as a 'reverse pharmacology' approach (Libert *et al.*, 1991) to distinguish it from more conventional drug discovery approaches. The conventional approach was historically initiated by the discovery of a biological activity for which the ligand responsible was identified and then used to characterize tissue pharmacology and physiological role. Subsequently, the ligand was used to clone its corresponding receptor for use as a drug target in high-throughput screening. The reverse approach starts with an orphan receptor of unknown function which is used as a 'hook' to fish out its ligand. The ligand is then used to explore the biological and patho-physiological role of the receptor. High throughput drug screening is initiated in parallel to develop tool compounds to help determine the therapeutic value of both agonists and antagonists to the receptor.

Screening strategy

Figure 1 illustrates the reverse pharmacology strategy adopted within SmithKline Beecham. The majority of our orphan receptors have been identified through extensive bioinformatic analysis of expressed sequence tag (EST) databases generated by mass random sequencing of cDNA libraries. The EST approach has previously proven to be a highly productive route for identifying novel genes (Adams *et al.*, 1992).

Since cDNAs identified by EST sequencing are often incomplete, the tissue expression pattern of the EST is analysed *via* Northern blot or RT–PCR to identify the tissue cDNA libraries which should be used to obtain a full length clone. More significantly, the expression pattern can determine whether a receptor is expressed in a normal or diseased tissue of interest as a therapeutic target. A highly selective tissue expression profile can also provide a clue to receptor function. For example, the expression pattern of the orphan receptor FC5 in rat brain as determined by *in situ* hybridization was recognized as resembling that of the Y_1 NPY receptor visualized by ligand autoradiography and hence led to the identification of FC5 as the Y_5 receptor. (Eva *et al.*, 1990). In parallel with obtaining a full length cDNA for the receptor, corresponding genomic clones can be obtained and their sequences from different individuals analysed to look for genetic markers that can be used to investigate possible associations with disease states.

Once a full length cDNA is available it can be expressed in mammalian cell lines for functional analysis. The choice of expression system is crucial to the success of ligand fishing: a system with a good history of GPCR expression which also contains a wide repertoire of G-proteins to allow functional coupling to down-stream effectors is of key importance. CHO (Chinese Hamster Ovary) or HEK (Human Embryonic Kidney) 293 cell lines are often the cells of choice, but as the success of receptor expression and coupling cannot be predicted, a variety of systems may have to be used. Alternative expression systems which can be used to explore different coupling mechanisms include xenopus oocytes, melanophores (Lerner, 1994) and engineered yeast systems (Brouch & Thorner, 1996).

In the absence of a ligand to confirm receptor expression it is important to obtain some evidence that receptor transfections have been successful before embarking on the search for a ligand. Northern blotting of cell lines is probably the easiest test, but will only confirm that message for the receptor is present. To be confident that receptor protein is actually expressed one has to generate antibodies to the receptor or else attach an epitope tag to the receptor and assess protein expression *via* FACS analysis or Western blotting. A number of epitope tags have been successfully used to label GPCRs without affecting receptor function. Examples include FLAG and HA tags, for which antibodies are commercially available. (Guan *et al.*, 1992; Koller *et al.*, 1997).

The choice of functional assays used to screen for activating ligands is also critical to the success of ligand fishing. These should be as generic as possible to allow detection of a wide range of coupling mechanisms. Measurement of metabolic activation of cells expressing the orphan *via* the Cytosensor microphysiometer (McConnell *et al.*, 1992) is probably the most generic assay available, but is

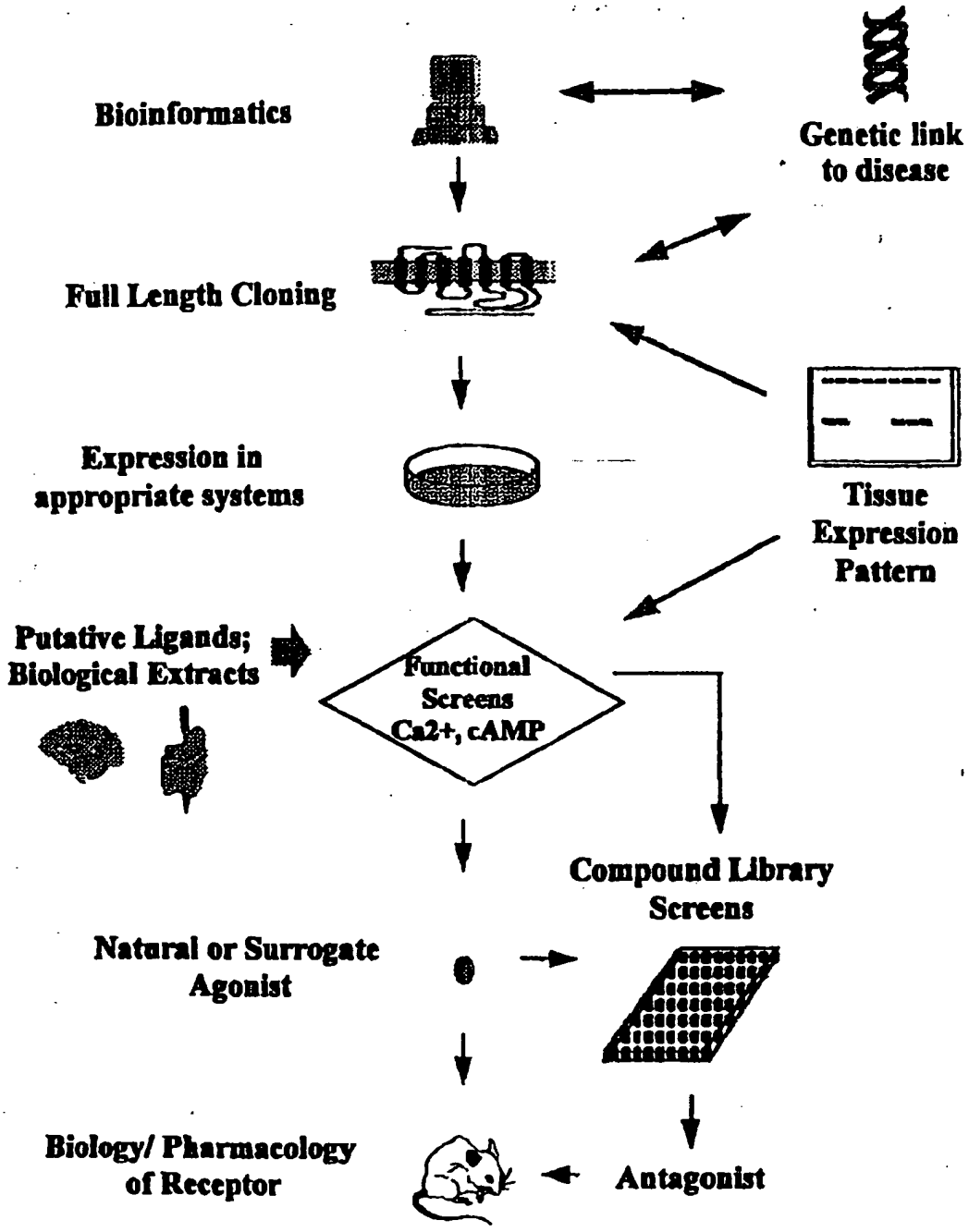

Figure 1 Reverse pharmacology approach to characterizing orphan GPCRs (modified from Stadel *et al.*, 1997).

hampered by its low screening throughput. Alternative assay systems in mammalian cells focus largely on measuring changes in intracellular cyclic AMP or Ca^{2+} levels, either directly using standard methods or *via* the use of reporter gene assays. It is becoming increasingly important to use high throughput systems to allow screening of large libraries of ligands, and assay technologies have evolved for each of these second messenger systems to allow high throughput readout in microtitre plate format. More recently, it has become possible to funnel heterologous GPCR signal transduction through a common pathway involving phospholipase C and Ca^{2+} mobilization by co-expression of the receptor with the promiscuous G-proteins $G_{\alpha 15/16}$ or with chimeric G_q-proteins such as G_{qi5} (Conklin *et al.*, 1996; Offermans & Simon, 1995). Although this approach may not work universally, the diversity of known GPCRs reported to successfully couple *via* these G-proteins suggests that it is a useful method to streamline screening for orphan receptor activation by focusing predominantly on one signal transduction system.

One factor which can complicate the use of heterologous expression systems for ligand fishing involves the presence of endogenous receptors in mammalian cell lines and in particular, clonal variation in the pattern of endogenous receptor expression in cells derived from the same parental cell line. Such variation has probably been responsible for the mis-

identification of a number of orphan receptors in the past. (eg. Cook *et al.*, 1992; Jazin *et al.*, 1993).

The ability to genetically delete endogenous GPCRs from yeast to generate a 'null' background is one of the major advantages in using yeast model systems for orphan receptor screening (Broach & Thorner, 1996). These systems rely on commandeering the endogenous yeast GPCR transduction system, the pheromone mating pathway, to allow coupling of transfected human GPCRs and humanized G-proteins to the endogenous signalling machinery. The manipulations involve conversion of the normal yeast response to pheromone activation (growth arrest) to positive growth on selective media, or to reporter gene expression. Such systems provide rapid, high throughput means of screening orphan receptors.

Once expression of the receptor has been achieved in mammalian or yeast systems and functional assays are in place, the search for activating ligands can begin. The receptors are screened initially against a bank of putative ligands, which includes known GPCR ligands as well as other naturally occurring bioactive molecules of unknown mechanism. The next step is to search for novel ligands by screening biological extracts obtained from tissues, biological fluids and cell supernatants. Another option is to screen peptide or compound libraries for 'surrogate' agonists that can be used as tool compounds. Yeast model systems here again provide unique advantages, in that they can be engineered to express and secrete small peptides from a random peptide library that will permit autocrine activation of heterologously expressed receptors, thus allowing a facile readout for detecting surrogate agonists of the receptor. Once an activating ligand has been found, whether natural or surrogate, it can then be used to explore the biology of the receptor *in vivo* or in tissue preparations, and can also be used to configure a high throughput screen to search for antagonists to support biological studies.

Characterization of orphan receptors

Examples are now emerging of the success of the reverse pharmacology approach in identifying orphan GPCRs. A first example is the identification of a calcitonin receptor-like orphan as a CGRP receptor. CGRP (calcitonin gene-related peptide) is a potent vasodilator widely distributed in central and peripheral neurones. It has also been implicated in migraine and non-insulin dependent diabetes. An EST derived from a human synovium library was used to clone a full length cDNA from human lung. Sequence motif analysis placed the receptor firmly within the secretin/VIP subfamily of receptors, with its closest homologue being the calcitonin receptor. However, the message for this novel receptor was expressed predominantly in lung, known to be a source of CGRP receptors. Following expression in HEK293 cells, the receptor was screened against putative ligands using a cyclic AMP stimulation assay (the primary signal transduction pathway for the secretin/VIP receptor family) and was shown to respond to CGRP with high potency (EC_{50} 0.9 nM) (Aiyer *et al.*, 1996).

The pharmacological profile of the receptor determined in the cyclic AMP assay and in radioligand binding assays was subsequently shown to be similar to that observed with endogenous CGRP receptors in human neuroblastoma cells, indicating that the orphan receptor cDNA encoded a CGRP receptor. In contrast, other workers who cloned this receptor failed to detect a CGRP response following expression of the cDNA in COS cells (Fluhmann *et al.*, 1995). The explanation for this discrepancy was initially thought to be due to lack of appropriate coupling machinery for the receptor in COS cells, as we also failed to detect a functional response with the receptor in this cell line. However, it has been shown more recently that accessory proteins appear to be necessary to allow this receptor to show CGRP responsiveness, so an alternative explanation may lie in differential expression of such accessory proteins in HEK293 vs COS cells (McLatchie *et al.*, 1998). Whatever the explanation, these findings illustrate that the choice of expression system is an important factor in identifying ligands for orphan receptors.

A second example of successful orphan receptor characterization was the identification of the receptor for the anaphylatoxin C3a (Ames *et al.*, 1996). The anaphylatoxins C3a, C4a and C5a are potent inflammatory mediators released during complement activation and they have been implicated in a number of inflammatory diseases. An EST derived from a human neutrophil cDNA library was used to clone an orphan GPCR from the same library. The receptor showed low homology to known GPCRs, with the best match being to the C5a receptor (37% nucleotide identity). However, Northern blot analysis indicated that its expression profile was distinct from that of the C5a receptor. Functional characterization of the C5a receptor was known to be problematic in cell lines such as HEK293 and CHO, but the receptor was known to be functionally active when expressed in RBL-2H3 cells (a rat basophilic cell line).

The orphan receptor was therefore expressed in RBL-2H3 cells and screened against putative ligands in a Ca^{2+} mobilization assay. It produced a robust Ca^{2+} mobilization response to C3a but not to C5a or other ligands. In parallel transfections in the same cell line the C5a receptor was shown to elicit a robust Ca^{2+} response to C5a but not to C3a. Radioligand binding studies using [^{125}I]-C3a confirmed that the orphan receptor bound C3a with a KD of 0.3 nM. These findings, together with the tissue distribution data, are consistent with this orphan encoding the human C3a receptor (Ames *et al.*, 1996).

CGRP and C3a constitute examples where known ligands have been paired with an orphan receptor. A third example involves the pairing of a novel ligand with an orphan receptor: the identification of a novel neuropeptide as an endogenous ligand for the receptor GPR10. The human orphan receptor GPR10 was first reported by Marchese *et al.* (1995), who cloned the receptor from genomic DNA using a PCR-based approach. Like the majority of orphan receptors, GPR10

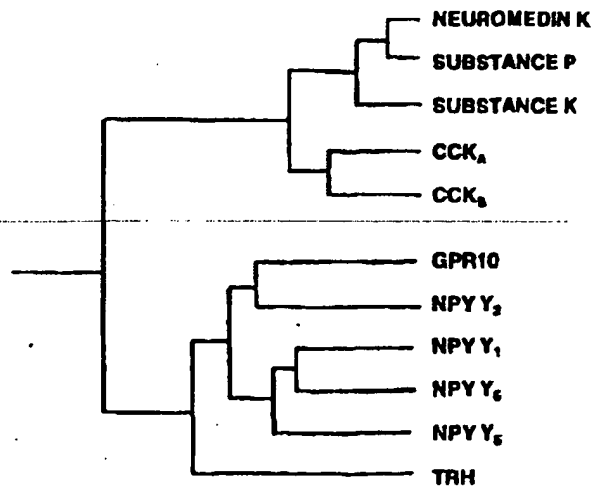

Figure 2 Phylogenetic tree for GPR10 and closely related receptors.

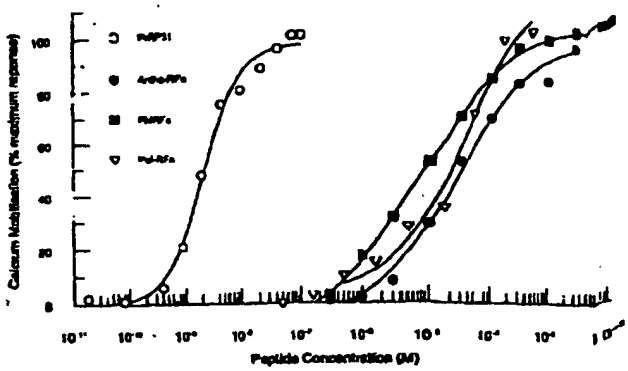

Figure 3 Ca^{2+}-mobilization responses to -RFamide peptides in HEK 293 cells stably expressing GPR10. EC_{50} values: PrRP31: 2 nM, *Antho*-RFa: 24 μM; FMRFa: 45 μM; *Pol*-RFa: 40 μM.

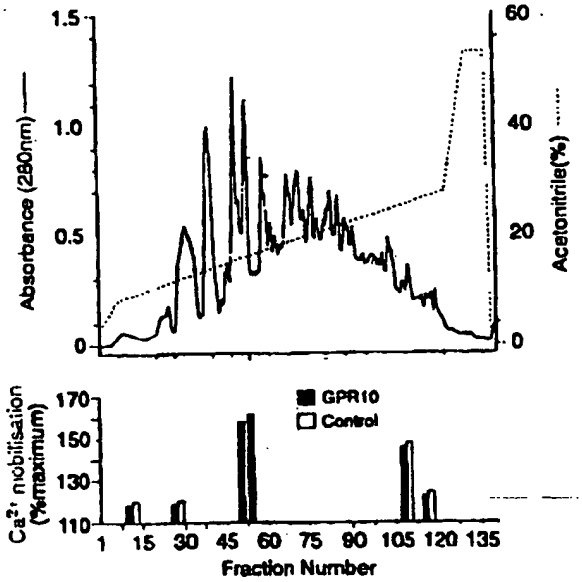

Figure 4 Stimulation of GPR10 by rp-hlpc fractions of crude peptide extracts of hypothalamus. Top panel, chromatogram. Acetonitrile gradient and absorbance of eluted materials indicated by dotted and solid lines respective. Bottom panel. Ca^{2+} responses in HEK293 cells stably expressing GPR10 or an unrelated orphan receptor (control). Peak Ca^{2+} responses were normalized to the maximum response induced by muscarine in the same cells. Note that a number of fractions induced similar responses in both cell lines, presumably acting through an endogenous receptor in HEK293 cells.

shows a low level of sequence homology with known GPCRs, although its closest match is to members of the NPY family of receptors (Figure 2). When expressed in HEK293 cells and screened against a bank of putative ligands, we found that the receptor failed to respond to NPY, but did produce robust Ca^{2+} mobilization responses to a number of short peptides found in invertebrates which are all members of the structurally related -RFamide peptide family (Figure 3). These peptides include FMRFamide, found in a variety of mollusc species, pQGRFamide (antho-RFamide) from *Anthopleura* and pQLLGGRFamide (pol-RFamide) from *Polycelis* (Greenberg & Price, 1992).

The potency of these peptides at GPR10 was low (EC_{50} values in μM range–see Figure 3); moreover, the two known mammalian members of the -RFamide family, NPAF and NPFF, were inactive at GPR10. We hypothesized that the natural human ligand for GPR10 would be a novel member of the -RFamide family, perhaps a longer peptide which retained the characteristic -RFamide C-terminal motif. We therefore set out to purify the peptide from biological extracts of tissues.

High resolution reverse-phase HPLC fractions of a porcine hypothalamus extract were screened against GPR10 stably expressed in HEK293 cells and specific, robust Ca^{2+} mobilization responses were found in adjacent fractions (Figure 4). The activities appeared to be peptidic, as protease treatment of the fractions destroyed activity. In a parallel but unrelated effort, Hinuma *et al.* (1998) used very similar methods to also identify specific, peptidergic activity in hypothalamic extracts screened against GPR10 expressed in CHO cells, using arachidonic acid release as the functional assay. The activity was purified to homogeneity by further rounds of HPLC and ion exchange chromatography, and sequencing revealed a 31 amino acid peptide that, as predicted, contained a C-terminal -RFamide motif: SRAHQH:MEIRTPDINPAWYAGRGIRPVG-RFamide. Hinuma *et al.* (1998) also found a second, overlapping peptide of 20 amino acids that appeared to be a truncated version of the longer peptide: TPDINPAWYAGRGIRPVG-RFamide. Cloning of the cDNA for the peptide revealed the existence of a prepropeptide containing an N-terminal secretory signal, and confirmed the sequence of the peptide (Hinuma *et al.*, 1998). The authors found that the longer peptide stimulated prolactin release from pituitary cells *in vitro*, and termed the peptides PrRP31 and PrRP20 respectively. PrRP31 shows high potency at GPR10 (EC_{50} 2 nM–see Figure 3), contrasting the low potency of the peptides from invertebrates, and suggesting that this peptide is the endogenous mammalian ligand for GPR10. The role of the peptide *in vivo* is under evaluation.

GPR10 is the most recent example of the identification of a novel ligand for an orphan receptor using the reverse pharmacology approach. A second example involved the discovery of the orexins (orexin-A and orexin-B), two related peptides derived from the same precursor by proteolytic processing. Both peptides were purified from brain extracts by screening for Ca^{2+} mobilization responses against an orphan receptor expressed in HEK293 cells (Sakurai *et al.*, 1998). Orexin-B showed significantly lower potency than orexin-A at the orphan receptor, termed OX_1, which subsequently led to the discovery of a second orexin receptor (OX_2) at which both peptides were equi-potent. The orexin peptides are expressed in the hypothalamus and appear to be involved in the regulation of feeding (Sakurai *et al.*, 1998).

Novel non-peptide ligands have also been identified for orphan receptors, examples being anandamide and 2-arachidonyl glycerol as putative native ligands for cannabinoid receptors (Devane *et al.*, 1992; Stella *et al.*, 1997). As more orphan GPCRs are discovered and subjected to reverse pharmacology approaches, it seems likely that the number of novel ligands identified will increase. Moreover, as the biology of novel ligand/receptor pairs is evaluated and their association with disease states explored, we believe they will yield new drug targets that will allow us to treat a range of as yet unmet medical needs. As a consequence, orphan GPCRs may well provide the pharmaceutical industry with the next generation of drug targets (Stadel *et al.*, 1997).

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